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UBIQUITIN AND THE CONTROL OF PROTEIN FATE IN THE SECRETORY AND ENDOCYTIC PATHWAYS[1]

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ABSTRACT

The modification of proteins by chains of ubiquitin has long been known to mediate targeting of cytosolic and nuclear proteins for degradation by proteasomes. In this article, we discuss recent developments that reveal the involvement of ubiquitin in the degradation of proteins retained within the endoplasmic reticulum (ER) and in the internalization of plasma membrane proteins. Both luminal and transmembrane proteins retained in the ER are now known to be retrotranslocated into the cytosol in a process that involves ER chaperones and components of the protein import machinery. Once exposed to the cytosolic milieu, retro-translocated proteins are degraded by the proteasome, in most cases following polyubiquitination. There is growing evidence that both the ubiquitin-conjugating machinery and proteasomes may be associated with the cytosolic face of the ER membrane and that they could be functionally coupled to the process of retrotranslocation. The ubiquitination of plasma membrane proteins, on the other hand, mediates internalization of the proteins, which in most cases is followed by

lysosomal/vacuolar degradation. There is, however, a well-documented case of a plasma membrane protein (the c-Met receptor) for which ubiquitination results in proteasomal degradation. These recent findings imply that ubiquitin plays more diverse roles in the regulation of the fate of cellular proteins than originally anticipated.

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INTRODUCTION

The ability to degrade proteins is an essential function of all eukaryotic cells. The two main proteolytic systems within eukaryotic cells are lysosomes and proteasomes. Lysosomes are membrane-bound organelles that contain an assortment of acidic hydrolases, including many proteolytic enzymes (Bohley & Seglen 1992). Lysosomes degrade macromolecules taken up from the extracellular medium and plasma membrane receptors down-regulated in response to ligand binding. They are also responsible for the turnover of most organellar proteins of the endocytic and late [post-endoplasmic reticulum (ER)] secretory pathways.

Proteasomes are multi-protein complexes found in the cytosol and the nucleus (reviewed in Coux et al 1996). Despite this localization, proteasomes do not

digest cellular proteins indiscriminately, but rather participate in the regulated breakdown of proteins that have been altered so as to be susceptible to degradation. As is discussed below, the most well-established means of targeting proteins to proteasomes is by their modification with chains of ubiquitin.

A simplistic view of cellular protein degradation thus would have the route of degradation determined in a topologically restricted manner, with lysosomes serving as the site for degradation of luminal and transmembrane proteins of the endocytic and secretory pathways, and proteasomes being responsible for ubiquitin-dependent degradation of cytosolic and nuclear proteins. However, recent developments indicate that the mechanisms involved in targeting proteins for degradation are substantially more complex than originally anticipated. In this review, we discuss observations in both yeast and mammalian cells that establish previously unappreciated roles for the ubiquitin-conjugating system in protein degradation from the ER and in the targeting of plasma membrane proteins for endocytosis and eventual degradation in lysosomes.

OVERVIEW OF THE UBIQUITIN-PROTEASOME DEGRADATION SYSTEM

Ubiquitin is a 76-amino acid polypeptide expressed in all eukaryotic cells and highly conserved from yeast to humans (reviewed in Wilkinson 1995). The covalent modification of proteins with chains of ubiquitin constitutes a potent targeting signal leading to recognition and destruction by the 26S proteasomes. The first physiological ubiquitination substrate was characterized in 1987 (Shanklin et al 1987); there are now at least 60 known substrates that include transcription factors, cell cycle regulators, kinases, phosphatases, tumor suppressors, and, as is described in detail below, a number of different transmembrane proteins. Importantly, for many of the substrates identified, ubiquitination occurs in a regulated manner, playing important roles in cellular processes for which regulation of protein levels are crucial (reviewed in Weissman 1997, Hershko & Ciechanover 1998).

Ubiquitination is an essential cellular process effected by a multi-enzyme cascade involving classes of enzymes known as E1s (ubiquitin-activating enzymes), E2s (ubiquitin-conjugating enzymes or Ubcs), and E3s (ubiquitin-protein ligases) (reviewed in Hochstrasser 1996, Weissman 1997, Hershko & Ciechanover 1998). A general scheme for ubiquitination is depicted in Figure 1. E1 activates ubiquitin in an ATP-dependent manner, with the formation of a thiol-ester linkage between the carboxy terminus of ubiquitin and E1. Sequential, transient thiol-ester bonds are then generated between the carboxy terminus of ubiquitin and specific cysteines of E2 and E3 enzymes. This “bucket brigade” of thiol-ester bonds culminates in the formation of an isopeptide linkage

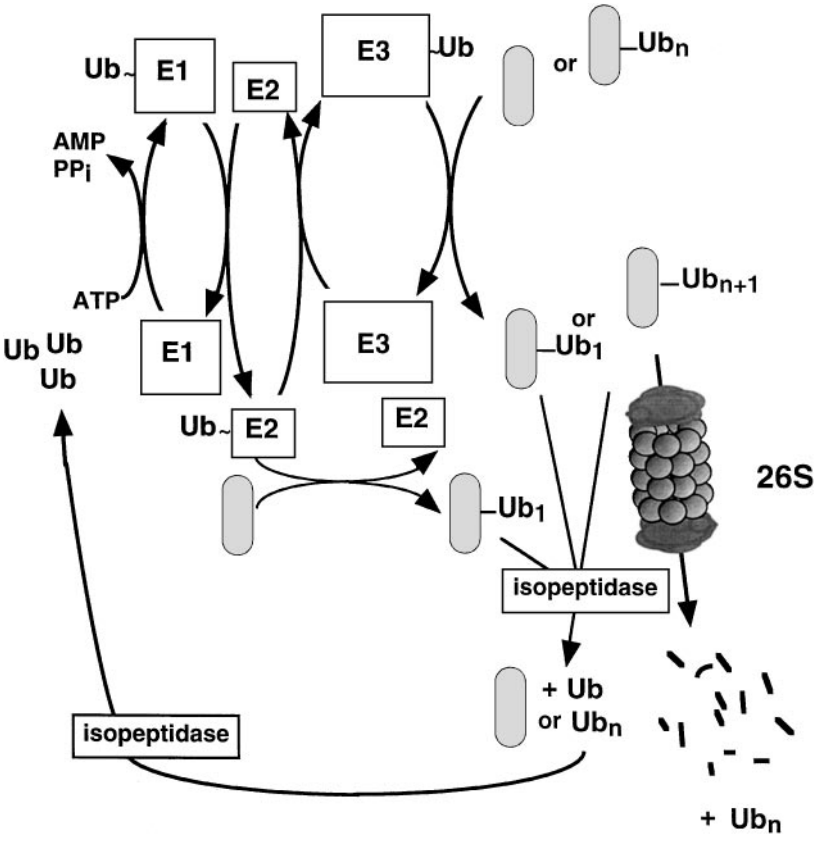

Figure 1 The ubiquitin-proteasome pathway. The components of the ubiquitin-conjugating system are shown schematically. Substrate is indicated by *shaded* object. Thiol-ester bond formation (~) between Ub and E3 is based on studies of HECT family E3s and may not universally apply. While E2s clearly can carry out mono-ubiquitination in the absence of E3s, it may be that in certain instances E2s can function in the polyubiquitination of substrates unassisted by E3s. The term isopeptidase is used to refer to any protein that cleaves linkages between the carboxy terminus of ubiquitin and the ϵ-amino group of a lysine residue. De-ubiquitinating enzymes therefore are members of the isopeptidase family.

between the activated carboxy terminus of ubiquitin and the ϵ-amino group of a lysine on a target protein or within another ubiquitin chain (usually K48 of ubiquitin), resulting in the generation of chains of ubiquitin generally referred to as either polyubiquitin or multiubiquitin (Figure 1). Although E1 serves a general purpose within the cell, specificity in substrate recognition lies largely at the level of E3s, with an additional degree of combinatorial specificity likely resulting from specific E2-E3 interaction. There is also evidence that E2s, either

singly or in pairs, function to at least mono-ubiquitinate proteins in the absence of E3s. Additionally, it should be noted that while some E3s form transient thiol-ester bonds with ubiquitin, there are other E3 activities where this may not occur and in which E3s serve primarily to bring E2s and substrates into proximity (reviewed in Weissman 1997, Hershko & Ciechanover 1998).

Two forms of E1 are distinguishable by SDS-PAGE: These are likely generated from use of alternative translation start sites in the E1 mRNA (Handley-Gearhart et al 1994a, Stephen et al 1996). The importance of E1, and of ubiquitination in general, is underscored by the cell cycle arrest exhibited by mammalian cells that express a temperature-sensitive E1 (Kulka et al 1988, Handley-Gearhart et al 1994b, Sudha et al 1995). This cell cycle arrest may be due to the now well-established role of the ubiquitin-conjugating system in degradation of cyclins and other molecules crucial to normal cell cycle progression (Weissman 1997, Hershko & Ciechanover 1998). In contrast to the paucity of distinct E1s, *Saccharomyces cerevisiae* expresses 12 distinct E2 enzymes, each of which is the product of a different gene; at least 17 E2s have been described in humans. E2s are characterized by a conserved 14–16-kDa core domain with amino- and carboxy-terminal extensions that provide substrate specificity or may allow for E3-independent ubiquitination (reviewed in Jentsch 1992, Hochstrasser 1996, Haas & Siepmann 1997). Despite the similarity among the various E2s, in multiple instances loss of a single E2 produces discernible phenotypes (Muralidhar & Thomas 1993, Harbers et al 1996, Roest et al 1996, Zhen et al 1996). E2s of particular relevance to this review are the core 16-kDa *S. cerevisiae* E2s—Ubc4p and Ubc5p—that are implicated in a number of cellular functions, and *S. cerevisiae* Ubc6p and Ubc7p. Ubc6p is unique among E2s in having a carboxy-terminal hydrophobic tail that allows for post-translational anchoring to the ER membrane (Sommer & Jentsch 1993, Yang et al 1997). Ubc7p is a soluble E2 that interacts functionally with Ubc6p and is recruited to the ER membrane by interactions with a newly described membrane-anchored protein, Cue1p (Chen et al 1993, Biederer et al 1996, 1997).

As already noted, E2s may directly donate ubiquitin to proteins. However, in most cases, the modification of proteins requires E3 activity (Weissman 1997, Hershko & Ciechanover 1998). Despite the clear importance of E3s, the characterization of this class of enzyme is in its infancy. The first E3 described at a molecular level was the *S. cerevisiae* enzyme responsible for the amino-terminal-dependent ubiquitination of proteins, a process canonized by Varshavsky and co-workers in the "N-end rule" (Varshavsky 1996). This E3 is known as Ubr1p; functionally related mammalian proteins, for which amino acid sequence is not yet available, have been termed E3α and E3β (Hershko & Ciechanover 1998).

Much of our knowledge of E3 enzymes has emerged from studies on p53 ubiquitination and degradation. This tumor suppressor is rapidly degraded

in cells expressing isotypes of the human papilloma virus (HPV) E6 protein from strains of this virus that predispose to malignant transformation (Scheffner et al 1990). Work from Howley and co-workers led to the discovery that E6 proteins from these strains (HPV16 and HPV18) serve as adaptors between p53 and a normal cellular protein known as E6-AP (E6-associated protein), allowing for the ubiquitination of p53 by E6-AP (Scheffner et al 1993). This led to the realization that the deduced amino acid sequences of a number of partially characterized cDNAs share carboxy-terminal homology with E6-AP, in a region implicated in the E3 activity of E6-AP (Huibregtse et al 1995). For this reason, these proteins have been termed HECT (homology to E6-AP carboxy terminus) proteins. It is now clear that several of these related proteins are enzymatically active E3s and, as will become evident, at least one member of this family plays a crucial role in the ubiquitination and the subsequent fate of plasma membrane proteins. There is also emerging evidence that a number of other structurally unrelated proteins, either singly or in combination, act as E3s. A recent example is the complex of Skp1p, Cdc53p, and Cdc4p, which has E3 activity in the ubiquitination of the Cdk inhibitor Sic1 (Feldman et al 1997). As proteins with E3 activity are identified, what it actually means to be an E3 will need to be re-evaluated (discussed in Weissman 1997).

The formation of K48-linked polyubiquitin chains on proteins constitutes a potent targeting signal for degradation in 26S proteasomes. These polyubiquitin chains are generated by isopeptide linkages between the ϵ-amino group of K48 of a ubiquitin moiety and the carboxy terminus of a newly added member of the chain. These K48-linked chains can be extensive in length (20 or more). It is generally accepted that a single ubiquitin on a protein, or single ubiquitins on multiple distinct lysines, are not sufficient to serve as a proteasomal targeting signal. The minimal chain length required for proteasome targeting has not been established with certainty, but appears to be no more than a chain of four ubiquitin moieties (Deveraux et al 1994, 1995). The extent to which polyubiquitin chains, linked through lysine residues other than K48, are generated in vivo or serve as proteasomal targeting signals remains an open issue.

The proteolytic component of the eukaryotic 26S proteasome is a 20S multicatalytic structure with trypsin-, chymotrypsin-, and postglutamyl hydrolase-like activities (reviewed in Coux et al 1996). This 20S structure consists of four stacks of seven subunits arranged in a barrel-like configuration with a hollow core (Lowe et al 1995, Groll et al 1997). Formation of the 26S proteasome occurs as a result of the addition of one or two multi-subunit 19S caps to ends of the 20S structure. These caps are notable for several features. First, at least one subunit capable of binding polyubiquitin chains has been discerned (S5a in human, MBP1 in *Arabidopsis thaliana*) (Deveraux et al 1994, 1995). Initial enthusiasm that S5a represents the unique ubiquitin recognition element has been tempered

by the observation that yeast cells deficient in the homologue of this subunit are viable, despite the clearly essential roles of ubiquitination and proteasomal degradation in *S. cerevisiae* (Coux et al 1996). Thus there may be other, yet to be determined, recognition elements for ubiquitinated proteins. Second, Doa4p, a subunit of the 19S cap in *S. cerevisiae*, has been shown to cleave polyubiquitin chains from residual peptides that remain proteasome associated after digestion of proteins (Papa & Hochstrasser 1993). Third, the 19S structure includes multiple subunits with ATPase activity. Although the range of activities of these subunits has not been exhaustively analyzed, they may be responsible for the unfolding of proteins as they are fed into the multi-catalytic proteasome core (reviewed in Coux et al 1996). Finally, other recognition elements exist within the 19S structure. In particular, the 19S cap recognizes the protein antizyme in a ubiquitin-independent manner. Antizyme complexes with ornithine decarboxylase, thereby targeting the latter for ubiquitin-independent proteasomal degradation (Bercovich et al 1989, Murakami et al 1992, Elias et al 1995, Li et al 1996).

Proteasomes are not static, fixed structures, but rather are subject to dynamic alterations in composition. For example, treatment of cells with γ-interferon results in increased incorporation of three subunits into the 20S multi-catalytic complex and the reciprocal loss of three other subunits. These alterations facilitate generation of peptides that, when transported into the ER, are efficiently loaded onto and presented at the cell surface by major histocompatibility complex (MHC) encoded class I molecules. Exposure to γ-interferon also results in the replacement of the 19S cap with the 11S regulator or PA28. This alternative proteasome component is not known to have a ubiquitin recognition element, but results in a particle believed to have enhanced overall activity, facilitating the generation of peptides appropriate for presentation by MHC class I molecules (reviewed in Coux et al 1996, Rock 1996, Weissman 1997).

Although the addition of polyubiquitin chains is a potent targeting signal for degradation in the 26S proteasome, it is also evident that ubiquitination is reversible and that a number of not fully characterized, de-ubiquitinating enzymes exist free in the cytosol (Zhu et al 1996, 1997; reviewed in Hochstrasser 1996, Wilkinson 1997). Underscoring this significance of de-ubiquitination is the recurrent observation that when proteasome function is inhibited, primarily non-ubiquitinated forms of proteins accumulate.

DEGRADATION OF ER-RETAINED PROTEINS BY THE UBIQUITIN-PROTEASOME SYSTEM

As mentioned above, most physiological substrates for proteasomal degradation identified to date are cytosolic or nuclear proteins (Coux et al 1996,

Weissman 1997). A major development in the past three years has been the realization that many proteins retained in the ER are translocated back into the cytosol and subsequently degraded by proteasomes. This discovery has shed light into the long-known but, until recently, poorly understood phenomenon of protein degradation from the ER (Bonifacino & Klausner 1994, Brodsky & McCracken 1997). Among the ER-retained proteins targeted for proteasomal degradation are abnormal newly synthesized proteins (e.g. mutant proteins or unassembled subunits of multi-protein complexes) and ER resident proteins, the levels of which are controlled by regulated proteolysis (e.g. HMG-CoA reductase) (Table 1). Although the final effectors of the degradation of these proteins are well-known components of the ubiquitin-proteasome machinery, the molecular mechanisms involved in translocating the proteins from the ER back to the cytosol are only now beginning to be unraveled. In the following sections, we review recent progress in the understanding of this process.

Import of Nascent Proteins into the ER

In order to understand how ER-retained proteins are targeted to the ubiquitin-proteasome system for degradation, it is first necessary to describe how proteins are initially imported into the ER. The vast majority of proteins destined for secretion into the extracellular space or for residence within compartments of the secretory pathway enter this pathway when they are co- or post-translationally translocated from the cytosol into the ER (reviewed by Corsi & Schekman 1996, Rapoport et al 1996). Both co- and post-translational passage of polypeptide chains through the ER membrane are mediated by the translocon, a cylindrical protein complex with a central pore. The basic structure of the translocon has been conserved throughout evolution; it consists of a complex (referred to as the Sec61p complex) of three integral membrane proteins named Sec61p, Sbh1p, and Sss1p in yeast, which correspond to Sec61α, Sec61β, and Sec61γ, respectively, in mammals (Corsi & Schekman 1996, Rapoport et al 1996). Sec61p/Sec61α is a multi-spanning membrane protein that constitutes the main protein translocation channel. An additional complex known as the Sec63p complex, composed of Sec62p, Sec63p, Sec71p, and Sec72p, is required for post-translational protein translocation. This complex is thought to bind newly synthesized proteins on the cytosolic side of the membrane and to transfer them to the Sec61p complex. Finally, there is evidence for the existence of a third complex composed of Ssh1p, Sbh2p, and Sss1p subunits that may be involved in the co-translational translocation of some proteins (Finke et al 1996). The ER luminal chaperone Kar2p (known as BiP in higher eukaryotes) binds to the luminal, DnaJ-like domain of Sec63p and, in an ATP-dependent manner, aids in the translocation process (Corsi & Schekman 1996, Rapoport et al 1996). Newly synthesized polypeptides containing hydrophobic transmembrane domains are

Table 1 Proteins retained in the ER that are degraded by the proteasome

Protein	Cause of ER retention-degradation	Evidence of ubiquitin involvement	References
α_1-antitrypsin	Mutant		(Qu et al 1996, Werner et al 1996)
α-GL-PLAP chimera	Mutation of GPI addition site		(Oda et al 1996)
Antithrombin	Mutant		(Tokunaga et al 1997)
ApoB	Limited availability of lipids	+	(Yeung et al 1996, Benoist & Grand-Perret 1997, Fisher et al 1997, Wu et al 1997)
CD4	Induced by HIV-1 Env and Vpu proteins		(Fujita et al 1997)
CFTR	Mutant, normal	+	(Jensen et al 1995, Ward et al 1995)
CPY (yeast)	Mutant	+	(Hiller et al 1996, Plemper et al 1997)
Cytochrome P-450s	Chemical damage, oxidation	+	(Tierney et al 1992, Roberts 1997, Yang & Cederbaum 1997)
HMG-CoA reductase (yeast)	Induced by mevalonate or its metabolites		(Hampton & Rine 1994, Hampton et al 1996)
MHC class I heavy chains	Induced by HCMV US11/US2 proteins or by DTT treatment, unassembled		(Wiertz et al 1996a,b, Hughes et al 1997)
p185c-erb B-2	Induced by geldanamycin		(Mimnaugh et al 1996)
Presenilin-2	Expressed in transfected cells	+	(Kim et al 1997)
Pro-α-factor (yeast)	Unglycosylated		(McCracken & Brodsky 1996, Werner et al 1996, Pilon et al 1997)
Proteinase A (yeast)	Mutant	+	(Hiller et al 1996)
Sec61p translocon subunit (yeast)	Mutant	+	(Biederer et al 1996)
Sss1p translocon subunit (yeast)	Mutation in associated Sec61p	+	(Biederer et al 1996)
TCR-α chain	Unassembled, differentiation-controlled in normal thymocytes	+	(Huppa & Ploegh 1997, Yu et al 1997, Yang et al 1998)
TCR-CD3-δ chain	Unassembled, differentiation-controlled in normal thymocytes	+	(Yang et al 1998, Bonifacino & Klausner 1994)
Tropoelastin	Brefeldin A-induced		(Davis & Mecham 1996)
Tyrosinase	Normal, increased in amelanotic melanomas	+	(Halaban et al 1997)

integrated into the ER membrane, whereas soluble polypeptides are delivered into the lumen of the ER.

Protein Modifications and Quality Control in the ER

As nascent polypeptide chains emerge in the ER lumen, they are subjected to a series of posttranslational modifications including signal peptide cleavage, addition of N-linked oligosaccharide chains, disulfide-bond formation, addition of

glycosyl-phosphatidylinositol anchors, folding, and assembly into oligomeric complexes. These maturation events are effected by the concerted action of ER-resident enzymes and molecular chaperones. Properly modified proteins then move to sites of vesicle budding and are transported to the Golgi complex. For most proteins, this intricate set of reactions is completed with astonishing efficiency. When proteins fail to undergo some of these modifications, however, they are generally retained in the ER (Hammond & Helenius 1995). Some ER-retained proteins are quite stable and, in the most extreme cases, accumulate in distended regions of the ER [e.g. Russell bodies containing abnormal immunoglobulins in myeloma cells (Valetti et al 1991)]. Other ER-retained proteins undergo rapid degradation from the ER (Bonifacino & Klausner 1994, Brodsky & McCracken 1997). The ability to discriminate between properly modified (i.e. normal) and improperly modified (i.e. abnormal) proteins, and the ensuing retention in the ER and degradation of abnormal proteins, have been referred to as the quality control function of the ER (Hammond & Helenius 1995). Quality control mechanisms are thought to serve an important physiological role by preventing the deployment of abnormal proteins at sites where they could potentially interfere with vital cellular functions.

Non-Lysosomal Nature of the Degradation of Proteins Retained in the ER

Over the past 10 years, numerous proteins have been shown to undergo degradation soon after translocation into the ER (for a comprehensive list, see Bonifacino & Klausner 1994). This phenomenon has been documented in a wide range of eukaryotic organisms, from yeast to humans, suggesting that it is the result of general, evolutionarily conserved processes. Early studies established some of the general characteristics of these processes (reviewed by Bonifacino & Klausner 1994, Brodsky & McCracken 1997). First, degradation from the ER was found to be highly selective, such that some proteins retained in the ER were rapidly degraded whereas others were not. Second, degradation was insensitive to inhibitors of lysosomal degradation in mammalian cells and unaffected by mutations of genes required for vacuolar degradation in yeast. Third, the proteins were last detected in the ER prior to degradation. Finally, degradation was not inhibited by blocking transport out of the ER-Golgi system with pharmacological inhibitors such as brefeldin A in mammalian cells or by mutation of genes involved in ER to Golgi transport in yeast (e.g. *SEC12*, *SEC17*, *SEC18*, or *SEC23*). The intriguing implication of these findings was that lysosomes, the main site of protein degradation within the secretory and endocytic pathways, were not involved in the degradation of proteins retained in the ER. Owing to these properties, the phenomenon became known as degradation from the ER, ER-associated degradation, or, simply, ER degradation.

Involvement of the Ubiquitin-Proteasome Pathway in the Degradation of Proteins Retained in the ER

The mechanisms involved in the degradation of proteins retained in the ER remained obscure for several years. One of the possibilities was that proteins were degraded by ER-resident proteases. However, despite some reports of proteolytic activities associated with ER fractions, efforts to identify specific ER proteases capable of complete, yet selective, protein degradation were unsuccessful. A major conceptual breakthrough occurred in 1993, when Sommer & Jentsch demonstrated that a defect in protein translocation into the ER caused by a mutation that destabilizes the Sec61p component of the translocon could be suppressed by mutation of the ubiquitin-conjugating enzyme Ubc6p (Sommer & Jentsch 1993). This observation suggested, for the first time, a connection between the degradation of an abnormal protein from the ER and the ubiquitin system. Then, in 1995, two groups reported that peptide aldehyde proteasome inhibitors (Rock et al 1994) and the structurally unrelated proteasome inhibitor lactacystin (Fenteany et al 1994) prevented degradation of both normal and mutant forms of the cystic fibrosis transmembrane conductance regulator (CFTR) in the ER (Jensen et al 1995, Ward et al 1995). This observation provided the first piece of evidence implicating proteasomes in the destruction of ER-retained proteins.

Additional experiments (Ward et al 1995) demonstrated that the proteasomal degradation of the CFTR required polyubiquitination of the protein. Treatment with proteasomal inhibitors, for instance, resulted in the accumulation of high molecular weight CFTR species that were recognized by anti-ubiquitin antibodies on immunoblot analyses (Ward et al 1995). Moreover, expression of a dominant-negative ubiquitin mutant (K48R), which prevents formation of polyubiquitin chains (Chau et al 1989), blocked degradation of both the normal and mutant forms of the CFTR (Ward et al 1995). In addition, decreased conjugation of ubiquitin and decreased degradation of the CFTR were observed in cells that carry a temperature-sensitive mutation of the ubiquitin-activating enzyme E1 at the nonpermissive temperature (Ward et al 1995). These observations firmly established a role for the ubiquitin-proteasome pathway in the degradation of the CFTR retained in the ER and raised the possibility that other ER-retained proteins could be degraded by the same pathway. This is now known to be the case, as many other proteins retained in the ER have been shown to be polyubiquitinated and degraded by a process sensitive to proteasome inhibitors (Table 1).

Export of Degradation Substrates from the ER to the Cytosol

The CFTR and other polytopic membrane proteins such as HMG-CoA reductase degraded from the ER have sizable portions of their polypeptide chains

exposed to the cytosol and are thus directly accessible to the ubiquitin-proteasome degradative machinery. In contrast, many other ER-retained proteins degraded by this pathway (Table 1) either have short cytosolic tails [e.g. the T-cell antigen receptor (TCR) α chain] or are completely sequestered within the ER lumen (e.g. α_1-antitrypsin mutants). This topology poses a problem of access because components of the ubiquitin-proteasome pathway are cytosolic. Obviously, such proteins must be transported back into the cytosol to be degraded by proteasomes.

The ER membrane is permeable to nascent polypeptide chains by virtue of the translocon (see above), as well as to peptides transported into the ER by an ABC-type transporter, TAP (Hill & Ploegh 1995). In both cases, however, the direction of the polypeptide flow is from the cytosol to the ER lumen. Although there was evidence for the release of partially translocated nascent chains from ER membranes in in vitro translation-translocation assays (Garcia et al 1988, Ooi & Weiss 1992) and for the release of a glycosylated tripeptide from the ER lumen in permeabilized yeast cells (Römisch & Schekman 1992), it was not known until recently whether these processes also occurred in live cells. Studies by Wiertz et al (1996a,b) demonstrated that this was indeed the case for MHC class I heavy chains synthesized in the presence of either the US2 or US11 proteins of human cytomegalovirus (HCMV). MHC class I molecules are complexes of a glycosylated type I integral membrane heavy chain bound to peptide and a soluble luminal protein, β2-microglobulin. US2 and US11 were found to cause dislocation of newly synthesized MHC class I heavy chains from the ER membrane to the cytosol in vivo, leading to their rapid destruction by a pathway sensitive to proteasome inhibitors. Fully folded and assembled MHC class I heavy chains could also be dislocated and targeted for proteasomal destruction in the absence of viral gene products by treatment of cells with dithiothreitol, a reducing agent that induces misfolding of proteins in the ER.

Like translocation of nascent chains into the ER, dislocation into the cytosol was expected to involve a proteinaceous channel that allows transfer of the polypeptide chains across the lipid bilayer. The work of Wiertz et al (1996b) showed that MHC class I heavy chains targeted for cytosolic destruction were transiently associated with the Sec61p complex, suggesting that dislocation is mediated by the translocon. Thus the release of ER proteins for cytosolic destruction seems to represent a reversal of the process by which nascent polypeptide chains are initially inserted into and translocated across the ER membrane.

Permeabilized Cell Systems and Yeast Genetic Approaches to Study Degradation of ER-Retained Proteins

In addition to the use of proteasome inhibitors, other approaches contributing to the explanation of the mechanism of degradation from the ER were the

reconstitution of the degradative process in permeabilized cells and the use of yeast genetic methodologies. McCracken, Brodsky, and colleagues (McCracken & Brodsky 1996, Werner et al 1996) developed a permeabilized yeast cell system that recapitulated some of the properties of the degradation process in vivo. In this system, newly synthesized, unglycosylated yeast prepro-α-factor, a soluble luminal protein, was released from the ER into the incubation medium and underwent degradation in a cytosol- and ATP-dependent fashion. The system proved particularly informative when used in combination with mutant yeast strains as sources of permeabilized cells or cytosol. Thus it was possible to establish that the ER-chaperone Cne1p/calnexin and the proteasome subunits Pre1p and Pre2p are involved in this process (McCracken & Brodsky 1996, Werner et al 1996).

Other yeast proteins such as HMG-CoA reductase and mutant forms of carboxypeptidase Y and proteinase A are also degraded from the ER (Hampton et al 1996, Hiller et al 1996). The availability of various yeast degradation substrates allowed the development of assays to examine the involvement of known gene products in the process, as well as to screen for novel components of the pathway. Among the known proteins that have thus been implicated in the process are ER chaperones (Cne1p/calnexin and Kar2p/BiP), the catalytic subunits of signal peptidase (Sec11p and Spc3p), components of the translocon (Sec61p and Sec63p), ubiquitin-conjugating enzymes (Ubc6p and Ubc7p), and subunits of the proteasome (Cim3p, Cim5p, Hrd2p, Pre1p, Pre2p, and Pre4p) (Table 2). One of the novel components identified through yeast genetic screens is Der1p, a small multi-spanning membrane protein localized to the ER (Knop et al 1996). Another novel component, Der3p/Hrd1p, has five putative transmembrane spans and a large carboxy-terminal domain oriented toward the ER lumen (Hampton et al 1996, Bordallo et al 1998). Finally, a third novel component of the pathway is Hrd3p, which is predicted to be a type I integral membrane protein with a large amino-terminal luminal domain (Hampton et al 1996) (Table 2). The functions of these latter three proteins, Der1p, Der3p/Hrd1p, and Hrd3p, have not been established.

An Integrated View of the Mechanism of Protein Degradation from the ER

The studies described above have begun to delineate the steps of the pathway by which ER-retained proteins are targeted for degradation by the proteasome. A hypothetical depiction of the pathway is shown in Figure 2. In this scheme, misfolded, unassembled or aberrantly modified proteins are recognized by ER chaperones such as Cne1p/calnexin or Kar2p/BiP or by other components of the protein processing or transport machineries (step 1, substrate recognition and targeting to the translocon). The translocon is then opened and reprogrammed

Table 2 Yeast mutants defective in degradation of proteins retained in the ER

Mutant	Protein	Probable role in ER degradation	References
cim3/sug1	Cim3p, subunit of the 19S proteasome particle	Substrate recognition in the cytosol and targeting to the 20S proteasome particle	(Hiller et al 1996)
cim5	Cim5p, subunit of the 19S proteasome particle	Substrate recognition in the cytosol and targeting to the 20S proteasome particle	(Hiller et al 1996)
cne1	Cne1p/calnexin, ER integral membrane chaperone	Substrate recognition in the ER? Targeting to the translocon?	(McCracken & Brodsky 1996)
cue1	Cue1p, cytosolically disposed, membrane-anchored protein localized to the ER	Binds Ubc7p	(Biederer et al 1997)
der1	Der1p, small hydrophobic protein localized to the ER membrane, four potential transmembrane spans	Substrate recognition in the ER? Targeting to the translocon? Retrotranslocation?	(Knop et al 1996)
der2/ubc7	Der2p/Ubc7p, ubiquitin-conjugating enzyme, interacts with Ubc6p, recruited to the ER membrane by Cue1p	Substrate ubiquitination	(Biederer et al 1996, Hiller et al 1996)
der3/hrd1	Der3p/Hrd1p, multi-spanning ER integral membrane protein with a luminally oriented domain containing a RING-H2 finger motif	Substrate recognition in the ER? Targeting to the translocon? Retrotranslocation?	(Hampton et al 1996, Bordallo et al 1998)
hrd2	Hrd2p, p97 component of the 19S proteasome particle, also known as TRAP-2	Substrate recognition in the cytosol and targeting to the 20S proteasome particle	(Hampton et al 1996)
hrd3	Hrd3p, luminally disposed ER integral membrane protein	Substrate recognition in the ER? Targeting to the translocon? Retrotranslocation?	(Hampton et al 1996)
kar2	Kar2p/BiP, ER luminal chaperone	Substrate unfolding in the ER? Retrotranslocation?	(Plemper et al 1997)

(Continued)

Table 2 *(Continued)*

Mutant	Protein	Probable role in ER degradation	References
pre1	Pre1p, component of the 20S proteasome particle responsible for its chymotrypsin-like activity	Proteolysis	(Biederer et al 1996, 1997, Hiller et al 1996, Werner et al 1996)
pre2	Pre2p, component of the 20S proteasome particle	Proteolysis	(Hiller et al 1996)
pre4	Pre1p, component of the 20S proteasome particle	Proteolysis	(Hiller et al 1996)
spc3	Spc3p, catalytic subunit of signal peptidase	Endoproteolytic cleavage in the ER lumen	(Fang et al 1997)
sec11	Sec11p, catalytic subunit of signal peptidase	Endoproteolytic cleavage in the ER lumen	(Mullins et al 1995)
sec61	Sec61p, pore-forming subunit of the translocon	Retrotranslocation	(Pilon et al 1997, Plemper et al 1997)
sec63	Sec63p, translocon subunit	Retrotranslocation	(Plemper et al 1997)
ubc6	Ubc6p, ubiquitin-conjugating enzyme anchored to the cytosolic face of the ER membrane, interacts with Ubc7p	Substrate ubiquitination	(Biederer et al 1996, Hiller et al 1996)

for retrotranslocation. Next the polypeptide chains are unfolded and forced through the translocon (step 2, retrotranslocation). Proteins such as Der1p, Der3p/Hrd1p, and Hrd3p can be involved in either of the two previous steps. Cytosolic chaperones (e.g. members of the Hsp70 and DnaJ families) can supply the force needed to drive the polypeptide chains through the channel. The retrotranslocated polypeptide chains either are released into the cytosol as soluble proteins or remain adhered to the cytosolic face of the ER membrane. N-linked oligosaccharides are removed from the polypeptides by a cytosolic N-glycanase, and the proteins are polyubiquitinated (step 3, release into the cytosol, deglycosylation, and polyubiquitination); the order of these three events has not been established. Polyubiquitinated proteins bind to the proteasome and are degraded to peptides; peptides are further broken down by cytosolic exopeptidases (step 4, proteasomal degradation). The entire process is likely to consume a large amount of energy because unfolding, retrotranslocation, ubiquitination, and proteasomal degradation are known (or expected) to be ATP

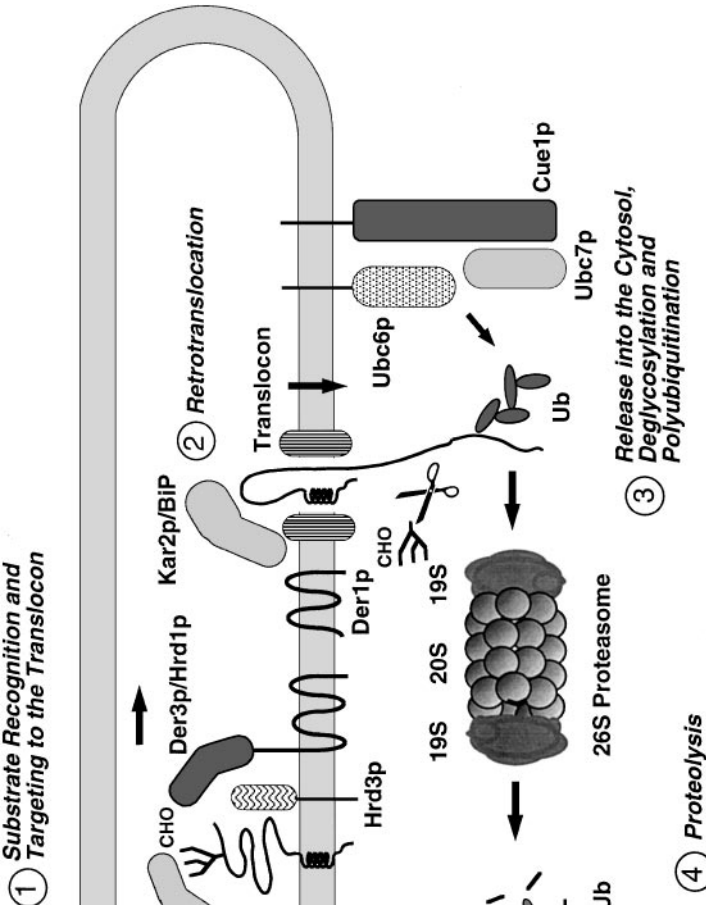

Figure 2 Schematic representation of the degradation of an ER-retained protein. See section "An Integrated View of the Mechanism of Protein Degradation from the ER" for description of the steps.

dependent. The steps of the process are discussed in more detail in the next four sections.

Recognition of Protein Substrates and Targeting to the Translocon

Quality control mechanisms are likely to involve sensing of the maturation state of newly synthesized proteins, a role probably played by the same proteins responsible for protein processing. For example, Kar2p/BiP has been shown to recognize specific sequences in folding or assembly intermediates (reviewed by Gething & Sambrook 1992). Similarly, Cne1p/calnexin binds monoglucosylated N-linked oligosaccharides that are intermediates in the processing of glycoproteins in the ER (Hammond & Helenius 1995). Thus whereas dynamic association with these ER chaperones may mediate folding and oligomerization of nascent proteins, failure to dissociate may promote interaction with the translocon, eventually leading to ejection of the proteins into the cytosol (see Knittler et al 1995). Analyses of yeast mutant strains have demonstrated that Kar2p and Cne1p are indeed required for the degradation of certain substrates by the ubiquitin-proteasome pathway (McCracken & Brodsky 1996, Plemper et al 1997).

In addition to misfolding, other features appear to predispose proteins for degradation from the ER. It is now clear that quality control mechanisms are capable of monitoring the trimming status of N-linked oligosaccharide chains and deciding the fate of a protein on this basis. For example, inhibition of ER mannosidases, which trim mannose residues from high-mannose, N-linked oligosaccharide chains, prevents degradation of unassembled TCR-CD3-δ chains of the T-cell antigen receptor (Yang et al 1998). This suggests that the ER may contain mannose-binding lectins that mediate recognition for degradation. Another feature known to destabilize proteins in the ER is the presence of short or unusually hydrophilic transmembrane domains, as is the case for the TCR-α and TCR-β chains (Bonifacino & Klausner 1994). When not assembled with other subunits of the complex, such transmembrane domains may remain associated with the translocon or with other transmembrane proteins in the ER, resulting in their rapid clearance from the ER. The reduced hydrophobicity of these transmembrane domains may also facilitate slippage of these proteins into the cytosol.

Certain viral gene products may target specific classes of proteins for degradation. For example, the HCMV US2 and US11 proteins are predicted to be transmembrane proteins with luminal domains that bind MHC class I heavy chains and transmembrane domains that might interact with the translocation machinery (Wiertz et al 1996a,b). The Vpu protein of human immunodeficiency virus-1 is another example of a viral protein that functions as a

specific recognition/targeting molecule, in this case for the CD4 co-receptor (Fujita et al 1997). ER proteins such as HMG-CoA reductase that are subject to metabolic control may interact with specific regulatory molecules that bring them to the translocation apparatus in response to metabolic stimuli. Finally, two of the transmembrane proteins newly identified through yeast genetic screens, Der3p/Hrd1p and Hrd3p, are predicted to have large luminal domains; these domains could participate in interactions with luminal substrates either before or during retrotranslocation.

Preparation of some proteins for retrotranslocation may entail limited proteolysis in the ER lumen. Signal peptidase has been shown to cause discrete fragmentation of certain abnormal proteins retained in the ER, prior to their complete degradation (Mullins et al 1995, Fang et al 1997). Interestingly, mutations in Sec11p and Spc3p, the subunits of signal peptidase required for catalytic activity, prevent both the fragmentation and the eventual destruction of chimeric proteins (Mullins et al 1995, Fang et al 1997), suggesting that signal-peptidase-mediated fragmentation may be an obligatory step in the targeting of those proteins for degradation in the cytosol. Indeed, cooperation between luminal cleavages performed by signal peptidase and degradation in the cytosol by the 26S proteosome has been suggested for the removal of mutant Sec61p from the yeast ER (Biederer et al 1996).

Retrotranslocation

MHC class I heavy chains targeted for destruction in the cytosol were shown to associate transiently with the mammalian Sec61p complex by co-precipitation with antibodies to its Sec61β or Sec61γ subunits (yeast Sbh1p and Sss1p, respectively) (Wiertz et al 1996b). In yeast cells, unglycosylated prepro-α-factor targeted for degradation was found to associate with Sec61p (the equivalent of mammalian Sec61α) (Pilon et al 1997). Taken together, these data suggest that the three basic subunits of the translocon are components of the complex involved in retrotranslocation. Strong support for these correlations comes from observations that some mutants in Sec61p are defective in retrotranslocation, thus implying that this protein is essential for export from the ER to the cytosol (Pilon et al 1997, Plemper et al 1997). In contrast, retrotranslocation is not affected by deletion of the gene encoding Sbh1p, indicating that other subunits of the translocon are not required for retrotranslocation even though they may interact with the translocating polypeptide chain (Plemper et al 1997). The degradation of mutant carboxypeptidase Y in yeast was not affected by mutations in Sec62p, Sec71p, Sec72p, Ssh1p, and Sbh2, all components of the protein import machinery (Plemper et al 1997). Mutations in Sec63p result in stabilization of mutant carboxypeptidase Y (Plemper et al 1997); however, other mutations in Sec63p that affect protein import have no effect in retrotranslocation of unglycosylated prepro-α-factor (Pilon et al 1997). A possible explanation for

these differences is that Sec63p may be necessary for export of some, but not all, substrates.

By analogy with the Sec63p complex, which adapts the Sec61p complex for post-translational import into the ER (Corsi & Schekman 1996, Rapoport et al 1996), there is likely a set of proteins that program the Sec61p complex for export from the ER. Perhaps this is one role played by the transmembrane proteins Der1p, Der3p/Hrd1p, and Hrd3p. Kar2p may promote retrotranslocation by effecting unfolding of the polypeptide chains. It is also likely that cytosolic chaperones such as hsc70 might bind to the polypeptides as they emerge on the cytosolic side of the membrane (Yang et al 1993).

Release into the Cytosol, Deglycosylation, and Polyubiquitination

Upon inhibition of proteasomal degradation, some retrotranslocated proteins are released as soluble proteins into the cytosol. These include luminal proteins such as unglycosylated yeast prepro-α-factor (McCracken & Brodsky 1996) and transmembrane proteins such as MHC class I heavy chains (Wiertz et al 1996a,b) and TCR-α chains transiently overexpressed in non-T cells (Huppa & Ploegh 1997, Yu et al 1997). Other proteins such as yeast carboxypeptidase Y (Hiller et al 1996) and a fraction of the transmembrane TCR-α in non-T cells adhere to the cytosolic face of the ER membrane (Huppa & Ploegh 1997, Yu et al 1997). For MHC class I heavy chains and TCR-α (Wiertz et al 1996a,b; Hughes et al 1997, Huppa & Ploegh 1997, Yu et al 1997), retrotranslocation is accompanied by the removal of N-linked oligosaccharides. Since the translocation pore is thought to be sufficiently wide to allow passage of complete N-linked oligosaccharides moieties attached to the polypeptide chains, and a cytosolic peptide:N-glycanase has been described (Suzuki et al 1994), it is likely that this deglycosylation occurs upon exposure to the cytosolic milieu.

Studies carried out in T lymphocytes on the TCR-CD3-δ chain have revealed another possible topology for proteins protected from degradation by inhibition of proteasome function. When the proteasome is inhibited, undegraded TCR-CD3-δ actually retains its normal transmembrane topology and remains dimerized with TCR-CD3-ϵ, which, unlike TCR-CD3-δ, is not normally targeted for degradation (Yang et al 1998). Along the same vein, when analyzed in its native context in T cells, TCR-α was found to undergo only partial retrotranslocation when proteasome function was inhibited, with the majority of the protein mass remaining sequestered within the ER lumen (Yang et al 1998). These observations suggest that the proteolytic activity of the proteasome is required for complete extraction of TCR-CD3-δ and TCR-α from the ER. Since both of these subunits are polyubiquitinated while still bound to the ER membrane (Yang et al 1998), it may be that polyubiquitination provides the physical link necessary for proteasome-dependent removal from ER membranes. Interestingly, TCR-α

lacks cytosolic lysine residues but has a number of luminal lysine residues, as well as one in the transmembrane domain (Chien et al 1984). Therefore, for this TCR subunit, initiation of retrotranslocation must precede polyubiquitination.

It is apparent that many, though apparently not all, proteins retrotranslocated into the cytosol become polyubiquitinated (Table 1). Biochemical and genetic analyses of the CFTR (Ward et al 1995), Sec61p and Sss1p (Biederer et al 1996) and mutant carboxypeptidase Y (Hiller et al 1996), have all demonstrated that polyubiquitination is required for degradation by the proteasome. Given that polyubiquitination is a prerequisite for the proteasomal degradation of many cytosolic and nuclear proteins, we anticipate that this will similarly be the case for other ER-retained proteins.

Attachment of polyubiquitin chains to degradation substrates is probably mediated by the ubiquitin-conjugating enzymes Ubc6p or Ubc7p, as mutation of genes encoding either of these enzymes in yeast prevents or retards degradation of proteins retained in the ER (Biederer et al 1996, Hiller et al 1996). Ubc6p is unique among ubiquitin-conjugating enzymes in that it is anchored to the ER and nuclear membranes by a carboxy-terminal hydrophobic tail, with its catalytic domain facing the cytosol (Sommer & Jentsch 1993, Yang et al 1997). The localization of Ubc6p to the ER may allow it to have immediate access to the retrotranslocated proteins, thus effecting rapid polyubiquitination of the proteins, perhaps while polypeptide chains are in the process of emerging from the translocon. Ubc7p is a soluble, cytosolic protein that is recruited to the ER membrane by interaction with a protein called Cue1p (Biederer et al 1997). Mutations in Cue1p abolish degradation of ER-retained proteins and, interestingly, prevent retrotranslocation as well, providing further evidence that ubiquitination and retrotranslocation are linked. The localization of Ubc6p and Ubc7p to the ER membrane raises the possibility that other components of the ubiquitin-proteasome pathway may be similarly associated with this membrane.

Western blot analyses of MHC class I heavy chains retrotranslocated by the US2 and US11 proteins have failed to reveal polyubiquitination when the proteasome is inhibited (Wiertz et al 1996a,b). Thus this protein may be an example of a group of proteins for which polyubiquitination is not required for targeting to the proteasome, as has been previously shown for the cytosolic protein ornithine decarboxylase (Murakami et al 1992). However, it is also possible that polyubiquitination of MHC class I heavy chains may not have been detected owing to either rapid deubiquitination by cytosolic deubiquitinating enzymes or to the quality of the antiubiquitin antibodies used for detection.

Proteasomal Degradation

Analyses of yeast mutants have implicated both the 19S and 20S particles of the 26S proteasome in the degradation of proteins released from the ER.

The involvement of the 19S particle in degradation was first suggested by the observation that a yeast gene involved in the regulated turnover of HMG-CoA reductase, HRD2, encodes a 109-kDa protein homologous to a component of the human 19S particle referred to as p97/TRAP-2 (Hampton et al 1996). Mutations in two additional subunits of the yeast 19S particle, Cim3p/Sug1p and Cim5p, also reduce the rate of degradation of ER-retained proteins (Hiller et al 1996). The 20S particle of eukaryotic proteasomes contains several catalytic subunits that hydrolyze peptide bonds with distinct site specificities (see above). The Pre1p subunit is responsible, at least in part, for the chymotryptic activity of the proteasome. Mutations in Pre1p alone, or in combination with mutations in two other proteasomal subunits, Pre2p or Pre4p, cause a marked decrease in the rate of degradation of ER-retained proteins (Biederer et al 1996, Hiller et al 1996, Werner et al 1996). Peptides produced by the proteasome escape from the central proteolytic channel through gaps between the proteasome subunits and are degraded by cytosolic exopeptidases.

Upon homogenization of cells, proteasomes are recovered mostly in the cytosolic fraction (Palmer et al 1996), suggesting that they exist free in solution. However, a subpopulation of proteasomes are bound to the cytosolic face of ER membranes (Rivett et al 1992, Palmer et al 1996). These membrane-bound proteasomes could be interacting with the Sec61p complex or with other proteins involved in retrotranslocation. Alternatively, proteasomes could be interacting directly with the lipid bilayer. In support of this notion, it has been demonstrated that proteasomes bind to lipid monolayers with the proteasome channel oriented perpendicular to the monolayer (Newman et al 1996). Membrane-bound proteasomes would be ideally disposed to degrade proteins as they emerge from the translocation apparatus. An attractive possibility, discussed above, is that proteasomes may even contribute to the extraction of ubiquitinated proteins from the ER membrane. Membrane-bound proteasomes could also be engaged in degradation of the cytosolic domains of integral membrane proteins prior to their extraction from the ER membrane. Evidence for this comes from in vitro experiments in which the 20S proteasome was found to degrade a cytosolically disposed segment of cytochrome P-450, leaving a fragment of the protein attached to the ER membrane (Roberts 1997).

Physiological Role of the Process of Protein Degradation from the ER

How essential is the ability to degrade proteins from the ER? Apparently, yeast cells can do very well without it. For instance, yeast cells bearing null mutations in the *HRD3*, *HRD1/DER3*, and *DER1* genes are viable and exhibit normal rates of growth in liquid culture (Hampton et al 1996, Knop et al 1996, Bordallo et al 1998). It remains to be determined, however, whether this

pathway is required for growth of yeast cells under conditions of stress. In mammalian cells, accumulation of undegraded abnormal proteins in the ER could potentially lead to cytopathic effects and cell death. This has been suggested as the underlying cause for liver disease in human patients, wherein abnormal forms of α_1-antitrypsin are synthesized (Amara et al 1992). In addition, the degradation of proteins from the ER is an integral part of mechanisms that regulate protein expression during development (e.g. the TCR complex) and under certain metabolic conditions (e.g. HMG-CoA reductase and apolipoprotein B) (reviewed by Bonifacino & Klausner 1994). It is thus likely that the ability to degrade proteins from the ER is essential in higher eukaryotes.

Another potentially important role for the degradation of ER-retained proteins in mammals is the production of antigenic peptides for presentation by MHC class I molecules. For instance, it has recently been demonstrated that dedifferentiation of melanoma cells causes tyrosinase, a type I integral membrane protein, to be retained in the ER and degraded by the ubiquitin-proteasome pathway (Halaban et al 1997). Interestingly, tyrosinase peptides produced in this way appear to be imported back into the ER and presented to the immune system by MHC class I molecules (Skipper et al 1996). It will now be of interest to determine whether other antigenic peptides derived from endogenous integral membrane or luminal proteins are also generated by this pathway.

UBIQUITINATION OF PLASMA MEMBRANE PROTEINS

In addition to the degradation of ER-retained proteins, ubiquitination has recently been implicated in the internalization and degradation of plasma membrane proteins. The first hints of a role for ubiquitin in the regulation of plasma membrane proteins were obtained in the mid-1980s, when several cell surface receptors, including the platelet-derived growth factor β-receptor (PDGFRβ) (Yarden et al 1986) and growth hormone receptor (GHR) (Leung et al 1987), were found to contain covalently attached ubiquitin chains. Also at that time, it was reported that the leukocyte homing receptor (Mel-14) was modified with ubiquitin on its intraluminal domain during its processing through the secretory pathway; the significance of this finding remains unclear (Siegelman et al 1986, St. John et al 1986). These initial observations occurred at a time when ubiquitination had been studied primarily as an in vitro process, and its role in general protein turnover was not fully appreciated. Beginning in 1992, studies involving multi-subunit receptors of the immune system and growth factor receptors revealed that ubiquitination of a number of cell surface receptors that signal through tyrosine kinase activation occurs in response to binding of ligands (Table 3).

Table 3 Transmembrane cell surface mammalian and yeast proteins that are subject to ubiquitination

Transmembrane protein	Characteristics (see below)	References
TCR	b,d,i	(Cenciarelli et al 1992, 1996, Hou et al 1994)
FcϵRI	b,j	(Paolini & Kinet 1993)
PDGFRβ	b,d	(Mori et al 1992, 1993)
c-kit	b,d,i	(Miyazawa et al 1994, AM Weissman unpublished data)
GHR	b,f	(Strous et al 1996, 1997)
EGFR	b,d	(Galcheva-Gargova et al 1995)
c-Met	b,g	(Jeffers et al 1997)
Fibroblast growth factor receptor	b	(Mori et al 1995a)
Colony stimulating factor 1 receptor	b	(Mori et al 1995a)
Insulin receptor	a,g,k	(Sepp-Lorenzino et al 1995)
Insulin-like growth factor receptor	a,g,k	(Sepp-Lorenzino et al 1995)
p185c-erbB-2	g,k	(Mimnaugh et al 1996)
Mel-14	l	(Siegelman et al 1986, St. John et al 1986)
ErbB-4	m	(Vecchi & Carpenter 1997)
Ste2p	b,e,f,h	(Hicke & Riezman 1996, Hicke et al 1998, Terrell et al 1998)
Ste6p	f	(Kolling & Hollenberg 1994, Kolling & Losko 1997)
Pdr5p	f	(Egner & Kuchler 1996)
Fur4p	c,f,h	(Galan & Haguenauer-Tsapis 1997, Galan et al 1996, Hein et al 1995, Marchal et al 1998)
Ste3p	b,f	(Roth & Davis 1996)
Gap1p	f,h	(Hein et al 1995, Springael & André 1998)

a, Ubiquitination not directly demonstrated; b, ligand-dependent ubiquitination; c, ubiquitin linkage analyzed; d, associated with tyrosine phosphorylation; e, associated with serine/threonine phosphorylation; f, ubiquitination associated with endocytosis; g, ubiquitination associated with proteasomal degradation; h, Rsp5 implicated; i, degraded primarily in lysosomes; ubiquitination not directly implicated; j, reversible ubiquitination; k, in response to benzoquinone ansamycins (herbimycin A, geldenamycin); l, luminal ubiquitination, significance unclear; m, ubiquitin-proteasome-mediated degradation of an intracellular cleavage product.

Ligand-Dependent Ubiquitination

One of the first plasma membrane receptors shown to be ubiquitinated upon ligand binding was the TCR (Cenciarelli et al 1992). These receptors are multi-subunit structures consisting of six distinct transmembrane polypeptides that, when appropriately assembled in the ER, are transported to the plasma membrane as an octameric complex ($\alpha\beta\gamma\delta\epsilon_2\zeta_2$) (reviewed in Weissman 1994,

Ashwell & Weissman 1995). The earliest requisite event in the pathway leading to T cell activation in response to TCR engagement is the activation of protein tyrosine kinases; these include Src family kinases and ZAP-70. Multiple TCR subunits were found to be ubiquitinated on cytosolic lysines in response to receptor occupancy (Cenciarelli et al 1992), and multiple lysine residues on the TCR-ζ subunit (which has nine intracytoplasmic lysines) were substrates for ubiquitination. Notably, lysines introduced into positions where they did not normally exist were similarly modified (Hou et al 1994). The high-affinity receptor for IgE (FcϵRI), like the TCR receptor, is a multi-subunit receptor ($\alpha\beta\gamma_2$) (Blank et al 1989). As with the TCR, the β and γ subunits of FcϵRI are rapidly ubiquitinated on ligation, and this ubiquitination is reversed when ligand is disengaged (Paolini & Kinet 1993).

In response to ligation, the PDGFRβ dimerizes with proximal sequelae that include activation of tyrosine kinase activity and autophosphorylation; this is followed by internalization and lysosomal degradation. Interestingly, PDGFRβ ligation also results in the appearance of high-molecular-weight ubiquitinated forms that are stabilized at 4°C (Mori et al 1992). Notably, a truncation of the carboxy terminus of this protein that substantially diminished detection of ubiquitinated forms had minimal effects on ligand-induced internalization (Mori et al 1993). Similar to PDGFRβ, the stem cell factor receptor, c-kit, undergoes ligand-induced ubiquitination (Miyazawa et al 1994). A number of other growth factor receptors have also been shown to be ubiquitinated in response to ligand (see Table 3).

Common to mammalian receptors that have been shown to undergo ligand-induced ubiqutination is a relationship between tyrosine kinase activation and ubiquitination. For the TCR, receptor occupancy in the absence of receptor-mediated activation of tyrosine kinases does not result in ubiquitination (Cenciarelli et al 1996). Inhibition of tyrosine phosphatase activity with resultant enhancement of receptor tyrosine phosphorylation correlates with increased levels of ubiquitinated forms for the TCR (Cenciarelli et al 1996). Similarly, for the PDGFRβ, mutations that destroy tyrosine kinase activity result in a loss of ligand-mediated ubiquitination (Mori et al 1993).

A key question regarding ligand-induced ubiquitination of plasma membrane receptors is whether this modification plays a role in receptor down-regulation by targeting to 26S proteasomes. For the TCR, the most effective agents in blocking degradation of the cell surface receptors are the lysosome inhibitors monensin and ammonium chloride (AM Weissman, unpublished observations). Proteasome inhibitors have minimal effects on occupancy-induced receptor degradation, and ubiquitinated forms do not accumulate to significant levels when T cells are treated with these reagents. We have obtained similar results with c-kit (AM Weissman, unpublished observations). For the PDGFRβ,

proteasomes have been alluded to as a means of degradation; however, the inhibition of degradation achieved with proteasome inhibitors is only partial (<20%), and the capacity of neither monensin nor ammonium chloride to inhibit ligand-mediated degradation has been assessed (Mori et al 1995b,c). Thus despite the fact that the TCR, c-kit, and PDGFRβ are all subject to ligand-mediated ubiquitination, for none is there convincing evidence that proteasomes play a major role in ligand-mediated degradation.

Observations made with the tyrosine-kinase-containing receptor c-Met clearly demonstrate at least one instance where a close relationship does exist between ubiquitination of a plasma membrane receptor and proteasomal degradation (Jeffers et al 1997). c-Met is composed of a 50-kDa extracellular α subunit and a 140-kDa membrane-spanning β subunit. On ligand binding, the β subunit is ubiquitinated and degraded in a proteasome-dependent manner. When proteasomes are inhibited, a 55-kDa intracellular cleavage product of the β subunit, which is normally labile and is generated as part of a shedding process, is also protected. These data suggest that for this receptor not only is ligand-mediated degradation a ubiquitin-proteasome-dependent process, but that the same pathway serves to degrade an intracytoplasmic fragment of this protein that has been freed from its normal physical and regulatory constraints. For the tyrosine kinase receptor ErbB-4, there is also evidence for ubiquitin-proteasome-dependent degradation of a proteolytic fragment; however, in this case ubiquitination is not implicated in the degradation of intact receptors (Vecchi & Carpenter 1997).

A Novel Role for Ubiquitination in Internalization

Although c-Met clearly exhibits proteasome-dependent degradation, in other instances such a relationship has not been established. If not for proteasomal degradation, what then is the role of ubiquitination of plasma membrane receptors? In considering this, we should bear in mind that ubiquitination represents a significant modification, each ubiquitin moiety adding 76 amino acids to a protein. If added to multiple lysines, it is hard to envisage that protein-protein interactions would not be markedly affected. Thus it is unlikely that ubiquitination of cell surface receptors is without functional consequences. One exciting clue to an alternative role for ubiquitination comes from a series of recent studies of yeast membrane proteins, demonstrating that for several receptors and transporters, ubiquitination is a targeting signal for endocytosis and subsequent degradation in vacuoles.

Ste2p is a G protein–coupled receptor that binds the yeast pheromone, α-factor. This receptor, like other G protein–coupled receptors, has seven membrane-spanning segments and a carboxy-terminal cytosolic tail that is subject to regulatory serine phosphorylation. In response to α-factor, Ste2p is rapidly

internalized and degraded. Hicke & Riezman (1996) have demonstrated that Ste2 accumulates in high-molecular-weight ubiquitinated forms at the cell surface in response to α-factor in endocytosis-deficient cells (*end4*). Three E2 enzymes, Ubc1p, Ubc4p, and Ubc5p, appear to be essential for viability in *S. cerevisiae* (Jentsch 1992). *ubc1ubc4* and *ubc4ubc5* mutants exhibited profound defects on Ste2p internalization that were not due to the general cellular consequences of these mutations (Hicke & Riezman 1996). These cells also exhibited a substantial reduction in α-factor-induced ubiquitinated forms. Ste2p degradation was also dramatically diminished in cells defective in vacuolar protease function (*pep4prb1*) but not in proteasome mutants (*pre1pre2* and *yta5*). These results establish a link between ligand-induced ubiquitination, endocytosis, and eventual targeting for vacuolar degradation. There are also additional data suggesting a role for ubiquitination in the relatively slow ligand-independent constitutive endocytosis of Ste2p (Hicke et al 1998).

Further insights into the relationship between ligand-mediated ubiquitination and endocytosis have been derived from an analysis of the carboxy-terminal tail of Ste2p, where evaluation of a region previously identified as crucial for endocytosis (SINNDAKSS, amino acids 333–339) led to the determination that this region is also required for ubiquitination (Hicke & Riezman 1996). When the cytoplasmic tail of Ste2p was truncated at amino acid 345, constitutive endocytosis and ubiquitination occurred in response to α-factor. However, when this was accompanied by mutation of the three S residues within the SINNDAKSS sequence to A, or of the K to R, both processes were largely curtailed; further analysis demonstrated that K337 is, at least within the context of the truncated receptor, a site of ubiquitination (Hicke & Riezman 1996). The finding that mutation of serine residues has effects on ubiquitination is reminiscent of findings in mammalian systems with IκBα (Alkalay et al 1995, Li et al 1995, Chen et al 1996) and of more recent studies with β-catenin, where serine phosphorylation plays important roles in ubiquitination (Orford et al 1997). It has recently been established that loss of a casein kinase I-like activity required for phosphorylation of the cytoplasmic tail also results in failure of Ste2p to undergo both ubiquitination and internalization (Hicke et al 1998).

The yeast **a**-factor receptor, Ste3p, like Ste2p, is a seven transmembrane segment G protein–coupled receptor. As with Ste2p, this protein undergoes both constitutive and ligand-induced internalization; however, it is distinguished from Ste2p by its rapid rate of constitutive internalization ($t_{1/2} = 20$ min). Ste3p undergoes constitutive mono- and di-ubiquitination, as well as phosphorylation (Roth & Davis 1996). Analysis of cells mutant for Ubc4p and Ubc5p or for ubiquitin has provided convincing evidence that constitutive Ste3p internalization is ubiquitin dependent. When endocytosis is blocked, increased levels of ubiquitinated species are detected, indicating that ubiquitination

precedes endocytosis. Because of its rapid constitutive turnover, evaluation of ligand-mediated endocytosis of wild-type Ste3p is problematic. To circumvent this, a truncated form of Ste3p, defective in constitutive endocytosis, was evaluated for ligand-dependent internalization. As with constitutive internalization, **a**-factor-dependent internalization is also ubiquitin dependent.

Ste6p is a member of the ABC family of transporters, responsible for secretion of the yeast mating pheromone **a**-factor. This transporter also accumulates at the plasma membrane in a ubiquitinated form in endocytosis-deficient mutants (Kolling & Hollenberg 1994). Consistent with a regulatory role for ubiquitination, the half-life of this protein was increased two- to threefold in *ubc4ubc5* double mutants. As with Ste2p, the half-life of this protein was most markedly increased in a strain in which vacuolar function was compromised (*pep4*). It was subsequently determined that the 117-amino acid linker joining the two halves of this ABC family transporter is important for ubiquitination and protein turnover. This linker region, known as the D-box, contains a sequence, DAKTI, reminiscent of the last five amino acids of the Ste2p SINNDAKSS sequence. As with Ste2p, removal of the region containing the DAKTI motif correlates with decreased ubiquitination and diminished endocytosis. Receptors bearing a lysine-to-arginine mutation within this sequence were modestly impaired in constitutive turnover but still capable of being substrates for ubiquitination (Kolling & Losko 1997). Thus the importance of this region is not due to the lysine residue serving as a potential site for ubiquitination. Transfer of the entire D-box to a heterologous stable membrane protein, Pma1p, resulted in endocytosis and a marked decrease in half-life, which correlated with Pma1p ubiquitination. However, transfer of a more limited region containing the DAKTI sequence did not effect such a change in stability. These results suggest that while the DAKTI region is important, it is by itself not sufficient to confer susceptibility to ubiquitination and endocytosis.

There are several other examples where a relationship between ubiquitination and endocytosis/vacuolar degradation of yeast multi-spanning membrane transporters has been established. These include the uracil permease (Fur4p) (Galan et al 1996), the yeast multidrug transporter (Pdr5p) (Egner & Kuchler 1996), and the general amino acid permease (Gap1p) (Springael & André 1998). For all of these, ubiquitinated species accumulate in mutants defective in internalization. Fur4p contains a destruction box (Galan et al 1994) similar to that found in the mitotic cyclins (Glotzer et al 1991). A point mutation within this region stabilizes this transporter from degradation (Galan et al 1994). In addition, Fur4p contains a PEST sequence near its amino terminus. PEST sequences rich in proline, glutamic acid, serine, and threonine are found in a number of soluble proteins that are rapidly degraded (Mansur & Androphy 1993). Mutation of serines to alanines within this region results in decreased

ubiquitination and degradation of Fur4p. The possibility that the significance of these sites is due to their being sites of phosphorylation is supported by the observation that mutations of these serines to glutamic acid, which mimics the charge of a phosphate residue, substantially restores turnover and ubiquitination to Fur4p (Marchal et al 1998).

The findings obtained with yeast receptors and transporters are leading to an expanded view of the role of ubiquitination in cellular function. It is now evident that ubiquitination is not just a targeting signal for proteasomal degradation but that it also plays an important role in targeting of plasma membrane proteins for ligand-dependent and constitutive internalization. How do the findings obtained in yeast relate to observations made in mammalian cells? In fact, they provide potential explanations for earlier observations where correlations were found between cellular E1 activity and stress-induced lysosomal degradation of proteins (Gropper et al 1991) and with maturation of autophagocytic vesicles (Lenk et al 1992). A role in trafficking within the endosomal/lysosomal system in mammalian cells may also help account for the abnormal accumulation of ubiquitinated species found in intraluminal inclusions in neurodegenerative disorders (Mayer et al 1991). To date, studies directly implicating ubiquitination in endocytosis and lysosomal degradation in mammalian systems have been limited to an analysis of the GHR in cells expressing a temperature-sensitive E1 (Strous et al 1996). In this analysis, GHR endocytosis was dependent on an intact ubiquitin-conjugating system, and GHRs were found to be degraded in lysosomes. Although this study established a correlation, no evidence has yet been provided demonstrating that it is the ubiquitination of GHR itself that is required for endocytosis.

Ubiquitin Linkages and Endocytosis

It is generally accepted that formation of polyubiquitin chains is required for targeting to and degradation by proteasomes. Studies on the TCR (Cenciarelli et al 1992) and FcϵRI (Paolini & Kinet 1993) have demonstrated that receptors may have a low valency of ubiquitin molecules attached. These findings, together with the known existence of multiple ubiquitination sites on TCR-ζ (Hou et al 1994), raise the possibility that these plasma membrane receptors are subject primarily to multiple mono-ubiquitinations. More recent studies on yeast proteins have added to our knowledge of ubiquitination linkages at the plasma membrane, and for several of these proteins, the predominant ubiquitinated forms appear to exhibit relatively few ubiquitins per protein (Roth & Davis 1996, Galan & Haguenauer-Tsapis 1997), compared with the high-molecular-weight smear often seen with proteins targeted for proteasomal degradation. In the case of Ste2p, truncated forms with only a single ubiquitination site exhibit a

low valency of ubiquitination, which is apparently sufficient for internalization (Hicke & Riezman 1996).

Genetic evidence suggests that mono-ubiquitination is sufficient for ligand-induced endocytosis of Ste2p (Terrell et al 1998). When ubiquitin is added to proteins or when polyubiquitin chains are generated, the carboxy terminus of ubiquitin condenses with the ϵ-amino group of a lysine through an isopeptide bond, and the amino terminus of ubiquitin itself is free. However, a chimeric molecule consisting of truncated Ste2p in tandem with ubiquitin in which the amino terminus of ubiquitin is contiguous with Ste2p supports internalization of Ste2p in the absence of any other ubiquitination events (Terrell et al 1998). These somewhat counterintuitive findings suggest that if there is a specific recognition event occurring at the plasma membrane involving ubiquitin, the structure recognized is substantially different from the polyubiquitin signal that leads to proteasomal degradation.

The nature of the linkage required for endocytosis of uracil permease (Fur4p) has also been explored, in this case by making use of a mutant yeast strain deficient in a proteasome-associated ubiquitin isopeptidase (Doa4p) (Papa & Hochstrasser 1993). This isopeptidase is responsible for the removal of ubiquitin chains from residual proteasome-associated peptides. When this enzyme is inactive, the levels of free ubiquitin available for conjugation are markedly reduced. In *doa4* cells, ubiquitination and endocytosis of Fur4p were rescued by overexpression of wild-type ubiquitin and also by expression of a mutant ubiquitin gene in which K48 was mutated (the classic site for polyubiquitin chain formation) but only partially rescued by a K63 mutant (Galan & Haguenauer-Tsapis 1997). These iconoclastic observations raise the possibility that for ubiquitination of Fur4p, K63 is a critical residue for ubiquitin chain addition.

Enzymes Implicated in Ubiquitination at the Plasma Membrane

As already discussed, ubiquitination is thought to involve the sequential action of three classes of enzymes. The E2s implicated in degradation from the ER, Ubc6p and Ubc7p, possess features that allow them to associate either directly or indirectly with the ER membrane. These characteristics may facilitate a role for these E2s in the ubiquitination of proteins undergoing retrotranslocation from the ER, perhaps even without an accompanying E3. In contrast, Ubc4p and Ubc5p, which are implicated in plasma membrane protein ubiquitination, are core E2s that lack characteristics predisposing them to being concentrated at the plasma membrane. Thus in modeling ubiquitination of transporters and receptors, it is reasonable to suspect the existence of E3-like molecules that can associate with the plasma membrane, and in fact there is accumulating

evidence for the involvement of such proteins in ubiquitination at the plasma membrane.

Studies on yeast uracil permease (Fur4p) (Hein et al 1995, Galan et al 1996), Gap1p (Hein et al 1995), and Ste2p (L Hicke, personal communication) have all revealed that an essential gene, *NPI1*, is involved in their endocytosis/degradation and that this gene encodes an E3 termed Rsp5 (Huibregtse et al 1995). Rsp5 is highly homologous to the fission yeast E3, Pub1 (Nefsky & Beach 1996) and to a mammalian HECT domain E3, Nedd-4 (Hatakeyama et al 1997, Kumar et al 1997), as well as to two other recently characterized human cDNAs that encode polypeptides containing HECT domains (Pirozzi et al 1997) (see below). All these proteins are characterized by multiple copies within their amino-terminal half of a tryptophan-based region known as a WW or Rsp5 domain (reviewed in Hofmann & Bucher 1995, Sudol et al 1995). WW domains associate with proline-rich regions (PXY) on proteins that are similar but not identical to regions recognized by SH3 domains. Additionally, Rsp5, Pub1, Nedd-4, and one of the human homologues all share an amino-terminal Ca^{2+}-dependent-lipid (CaL) binding domain first described for protein kinase C isoforms (Coussens et al 1986). Nedd-4 has been shown to translocate to the plasma membrane in response to increased intracellular Ca^{2+}, in a fashion dependent on this amino-terminal motif (Staub et al 1997). Although not yet directly implicated in ubiquitination at the plasma membrane, Nedd-4 is a binding partner for subunits of the amiloride-sensitive sodium channel in a yeast two-hybrid screen through a WW/PXY interaction (Staub et al 1996). The significance of this interaction has been verified by co-immunoprecipitation in mammalian cells. Notably, the Nedd-4 binding sites within the sodium channel subunits are deleted in Liddle's syndrome, an inherited form of hypertension characterized by failure to down-regulate these plasma membrane proteins (Hansson et al 1995, Tamura et al 1996).

Adaptor Molecules at the Plasma Membrane

A working model for ubiquitination of plasma membrane proteins is one in which proteins containing WW domains ubiquitinate a number of membrane and other cellular proteins, with specificity conferred by the relative affinity of different WW domains for cognate PXY motifs. However, unlike the amiloride-sensitive sodium channel, the primary amino acid sequence of a number of plasma membrane proteins that undergo ubiquitination, including Fur4p, and Ste2p, do not themselves encode PXY motifs. This suggests several possibilities: one is that there are other families of E3s involved in ubiquitination at the plasma membrane; alternatively there may be other, as yet to be discerned, interaction motifs contained within WW domain-containing E3s. Another possibility is that these E3s do not in all cases interact directly

with plasma membrane proteins, but rather they are brought into proximity of receptors by adaptor molecules. One candidate likely to fulfill such an adaptor role in mammalian cells is epidermal growth factor receptor (EGFR) -associated protein clone 15 (Eps15). This 100-kDa protein was first identified as a substrate for EGFR tyrosine kinase activity. Eps15 is a component of clathrin-coated pits, where it is localized to rims. Eps15 also interacts with the ear of α-adaptin, a component of the AP-2 clathrin adaptor complex (Benmerah et al 1995, 1996, Tebar et al 1996, Wendland et al 1996, van Delft et al 1997b). Interestingly, Eps15 is a substrate for mono-ubiquitination in response to EGFR ligation (van Delft et al 1997a) and contains within its carboxy terminus a proline-rich region with potential WW domain interaction sites. The yeast homologue of Eps15 is a protein known as Pan1p, which is essential both for receptor-mediated endocytosis and for internalization of lipids (Wendland et al 1996, Tang et al 1997). One can envisage that Pan1p/Eps15 recruits WW domain-containing E3s to the plasma membrane where they are brought into proximity of transmembrane proteins predisposed to ubiquitination.

PERSPECTIVES

The ubiquitin-conjugating system, once largely a subject of in vitro analysis, and more recently implicated in the proteasomal degradation of an increasingly long list of cytosolic and nuclear regulatory proteins, is now taking center stage in our evolving understanding of the means by which the fates of ER luminal proteins and of transmembrane proteins in the ER and beyond are controlled. The remarkable observations that have been made in the past few years are leading inexorably to the acceptance of the notion of bidirectional movement of proteins into and out of the ER. They also suggest previously unappreciated roles for translocons, chaperones, and oligosaccharides in the degradation of proteins from the ER and open exciting new vistas with links to clinical disorders

A major question that remains to be addressed is the overall significance of ubiquitination and proteasomes in extraction of proteins from the ER. In some cases, such as in the degradation of MHC class I molecules (Wiertz et al 1996a,b, Hughes et al 1997) and carboxypeptidase Y (Hiller et al 1996), ubiquitination is implicated either not at all, or only after proteins have been functionally denatured by removal from the ER membrane. In these instances, proteasomes basically serve to discard what has become cytosolic junk. In other cases, such as in the degradation of a temperature-sensitive mutant form of Sec61p, the ubiquitin-conjugating system is implicated in determining the fate of the protein while still in its native lipid environment (Biederer et al 1996). In the case of the degradation of some TCR subunits from the ER, not only does ubiquitination occur while still ER-membrane associated, but as a

consequence, proteasomes may be given the opportunity to provide the driving force for their removal from ER membranes (Yang et al 1998). It is clear that there are multiple variations on these themes. In some instances, ubiquitination might even be indirectly facilitating degradation from the ER in *trans*, as has been suggested for calnexin in the degradation of α_1-antitrypsin (Qu et al 1996). It would thus appear that the same chaperones that potentiate protein folding and oligomerization also serve to target proteins for degradation. If this is indeed the case, how do chaperones decide whether to move a protein onward or participate in its expulsion into the cytosol? Is this strictly a stochastic process, or are there specific recognition sites that determine the fate of nascent proteins? In the next few years, we can look forward to the identification of additional components involved in this process and to the development of a more integrated view of the mechanism and regulation of this important pathway.

On the cell surface, it is now clear that ubiquitination is unequivocally linked to endocytosis and subsequent lysosomal degradation of plasma membrane transporters and receptors. At first glance, it would appear that ubiquitination of lysosomally degraded plasma membrane proteins also serves as a degradation signal. However, it should be emphasized that, to date, ubiquitination has been implicated in internalization and only indirectly in lysosomal/vacuolar degradation. An obvious unaddressed question is how ubiquitin mediates endocytosis. A likely possibility is that ubiquitin binds to some component of the internalization machinery such as clathrin or the AP-2 adaptor complex in mammalian cells or the actin cytoskeleton in yeast cells (Marks et al 1997, Riezman et al 1996). Unlike yeast receptors and transporters, which tend to be delivered to the vacuole after internalization, a number of mammalian receptors undergo cycles of constitutive endocytosis and recycling or ligand-induced internalization, dissociation from ligand, and re-expression at the cell surface. Another important issue that needs to be addressed is the extent to which cycling of receptors correlates with rounds of ubiquitination and de-ubiquitination.

Although ubiquitination of plasma membrane proteins usually results in internalization, which in some cases is followed by lysosomal degradation, there is at least one example where ligand-mediated ubiquitination apparently targets a plasma membrane receptor (c-Met) for proteasomal degradation (Jeffers et al 1997). There are several examples of pharmacologically induced degradation of tyrosine kinase-containing plasma membrane receptors where ubiquitination and proteasomal degradation have been implicated (see Table 3). Having only just now addressed the topological conundrum of proteasomal degradation from the ER by invoking the involvement of the ER protein import machinery, we are once again faced with explaining how transmembrane proteins are targeted for proteasomal degradation, but this time either from the plasma membrane or from within the endocytic pathway. Stay tuned and watch as this story unfolds.

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